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Clinical Medicine Insights: Endocrinology and Diabetes

Age and Menopausal Status Affect Osteoprotegerin and Osteocalcin Levels in Women Differently, Irrespective of Thyroid Function

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ABSTRACT: Osteoprotegerin (OPG) and osteocalcin (OC) are essential bone proteins. Recent studies have demonstrated that they are not secreted solely by bone cells; they play roles in the vascular function and energy metabolism, and they are influenced by multiple factors. The aim of the current study was to investigate the influence of menopause and age on OPG and OC in women with different thyroid-stimulating hormone (TSH) levels.

MATERIAL AND METHODS: We studied 49 women with elevated TSH, 26 with suppressed TSH, and 67 age-matched euthyroid controls. Of them 64 were menstruating and 78 postmenopausal. Body weight, height, waist circumference (WC), body mass index (BMI), serum TSH, free thyroxin (FT4), OPG, and OC were measured.

RESULTS: Generally, both OPG and OC were higher in the postmenopausal women than in the menstruating subjects (OPG 3.85 ± 1.49 pmol/L vs. 5.84 ± 2.42 pmol/L, $P < 0.001$; OC 8.84 ± 3.70 ng/dL vs. 12.87 ± 6.45 ng/dL, $P < 0.001$), and within the two thyroid dysfunction subgroups and the controls (all $P < 0.05$). OPG correlated with age (postmenopausal rho = 0.57, $P < 0.001$; premenopausal rho = 0.31, $P = 0.015$). Among the premenopausal subjects, OPG was higher in those with low TSH than in the controls ($P = 0.048$). OC correlated negatively with BMI and WC in the postmenopausal group (Spearman rho = −0.25, $P = 0.03$ and rho = −0.42, $P < 0.001$ respectively). OC was higher in the postmenopausal subjects with low TSH than in those with elevated TSH ($P = 0.024$), and correlated positively with FT4 (rho = 0.40, $P = 0.002$) and negatively with TSH (rho = −0.29, $P = 0.013$).

CONCLUSIONS: In women, OPG and OC depended differently on age and menopause and, to a lesser extent, on the thyroid function and body composition.

KEYWORDS: osteoprotegerin, osteocalcin, menopause, thyroid

CITATION: Shinkov et al. Age and Menopausal Status Affect Osteoprotegerin and Osteocalcin Levels in Women Differently, Irrespective of Thyroid Function. *Clinical Medicine Insights: Endocrinology and Diabetes* 2014:7 19–24 doi:10.4137/CMED.S15466.

RECEIVED: March 16, 2014. **RESUBMITTED:** June 30, 2014. **ACCEPTED FOR PUBLICATION:** July 1, 2014.

ACADEMIC EDITOR: Nigel Irwin, Editor in Chief

TYPE: Original Research

FUNDING: The study was supported by grant no. 31/2006 of the Medical University of Sofia. The study was conducted, analyzed, and interpreted by the investigators independent of the industry sponsors.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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This paper was subject to independent, expert peer review by a minimum of two blind peer reviewers. All editorial decisions were made by the independent academic editor. All authors have provided signed confirmation of their compliance with ethical and legal obligations including (but not limited to) use of any copyrighted material, compliance with ICMJE authorship and competing interests disclosure guidelines and, where applicable, compliance with legal and ethical guidelines on human and animal research participants.

Introduction

Both osteoprotegerin (OPG) and osteocalcin (OC) are produced by the osteoblast (OB) cells, but they are regulated by different mechanisms and enter different metabolic pathways in the body. OPG is a decoy receptor of the receptor activator of nuclear factor kappa ligand (RANKL) and mainly acts to regulate osteoclast differentiation and activation.[1–3] It is a secretory glycoprotein, belonging to the Tumor necrosis factor (TNF) receptor superfamily. Though initially studied as a molecule regulating bone remodeling, it is now evident that it is produced in various tissues and organs, eg the skin, the liver, the stomach, the intestine, the lungs, and the vascular endothelium.[4] OPG has been implicated in the pathogenesis of atherosclerotic disease and has been thought to be a mediator of arterial calcification.[5] It may be both a marker of the atherosclerotic process in the vascular wall and a protective factor restricting atherogenesis.[6] Some authors view it as a possible marker of cardiovascular disease.[7] OC, on the other

hand, is a secretory glycoprotein, produced almost exclusively by bone OBs, though there are data that it may be secreted by other cell types as well.[8,9] Several recent papers have suggested an additional function of OC outside the bone tissue: modulating intermediary metabolism.[10–12] It seems that OC might be a direct regulator of glucose homeostasis, linking the skeleton with the intermediary metabolism, as reviewed by Kassi and Papavassiliou a couple of years ago.[13] It is also possible that OC and other bone-derived proteins undergoing carboxylation (bone matrix proteins) might be involved in the process of atherosclerotic vascular damage.[9] Both circulating OPG and OC levels are influenced by age and a variety of other factors. One of them, thyroid function, has a profound impact on bone remodeling and bone cell metabolism, and as such influences the serum concentrations of both molecules. OPG has been found to be increased in both hypothyroidism and hyperthyroidism and to change with the thyroid function.[14–16] OC, on the other hand, is also influenced by the levels of the thyroid hormone (TH) and the thyroid-stimulating hormone (TSH) and increases with hyperthyroidism, but decreases with depressed TH levels.[17] Bone metabolism changes with menopause and might, therefore, influence the OPG and OC levels. Botella-Carretero et al recently demonstrated that the menopausal status might be an independent factor determining the OPG levels irrespective of the thyroid function.[18] The aim of the current study was to investigate the influence of menopause and age on OPG and OC levels in women with different TSH levels who are not taking any TH-affecting medication.

Material

The participants were recruited from the cohort of a nationwide epidemiological screening study carried out by a team at the University Hospital of Endocrinology with the Medical University of Sofia. A total of 2,406 subjects were enrolled in a survey of thyroid disorders, cardiovascular morbidity, and diabetes in six areas in Bulgaria. Of these, 1,348 (56%) were female. All female subjects with elevated or suppressed TSH were assessed for inclusion in the present post-hoc analysis. Women with a history of current thyrotoxicosis or hypothyroidism who were receiving TH replacement or antithyroid medication were excluded. Estrogen or combined estrogen/progestin replacement therapy was an exclusion criterion as well, as were hypercalcemia or hypocalcemia. Forty-nine women (35%) with elevated TSH, and 26 (18%) with suppressed TSH, as found from the thyroid screening, met these criteria. Sixty-seven (67%) age-matched euthyroid women from the same cohort were included as a control group. As a result, a total of 142 female subjects were included in the current analysis. Of them 64 (47%) were menstruating and 78 (53%) were postmenopausal, defined as having passed at least 12 months after the last menstrual period (Table 1). None of the studied women were taking antiosteoporotic medication that could influence the OC and/or OPG levels at the time of the study. The bone mineral density of the study subjects was unknown, as was their vitamin D status since these were not included in the screening.

Methods

All participants signed an informed consent that had been approved by the local ethics committee, and filled in a structured questionnaire containing data on current disorders and medication, personal and family medical history, smoking, and menopausal status. Body height, body weight, and waist circumference (WC) were measured as recommended[19] and the body mass index (BMI) was calculated using the standard formula. The subjects were categorized by WC following the the International Diabetes Federation (IDF) criteria: normal WC up to 80 cm, and increased WC ≥80 cm. Fasting blood sample was collected for TSH, free thyroxin (FT4), OPG and OC measurement.

Ultrasensitive TSH was determined by a microparticulate immunoenzyme analysis (MEIA) using an automated

Table 1. Characteristics of the studied subjects. The data are presented as means ± SD, median and interquartile range, or proportions and 95% confidence interval. The Student *t*-test was applied for age, BMI, and OC, and Mann–Whitney *U*-test was used for OPG, WC, TSH, and FT4 to compare the menstruating and the postmenopausal groups. Abdominal obesity prevalence is expressed as the prevalence of subjects with increased WC.

	MENSTRUATING N = 64 (45%, 37–53)	PM N = 78 (55%, 47–63)	*P*
Age (y)	39.7 ± 7.8	61.0 ± 10.0	<0.001
BMI (kg/m^2)	24.95 ± 4.38	27.44 ± 4.92	0.002
Waist (cm)	77 (70.5–88.5)	89 (81–95)	<0.001
OPG (pmol/L)	3.61 (2.94–4.38)	5.3 (4.36–6.11)	<0.001
OC (ng/dL)	8.84 ± 3.70	12.87 ± 6.45	<0.001
TSH (mU/L)	2.49 (0.69–4.57)	4.50 (0.87–8.83)	0.013
FT4 (pmol/L)	12.5 (10.6–13.6)	10.9 (9.2–13.9)	0.097
Abdominal obesity n (%, 95% CI)	27 (42%, 30–54)	54 (69%, 58–80)	0.002

Abbreviation: PM, postmenopausal.

analyzer, AxSYM (ABBOTT, USA). The analytical sensitivity of the method was 0.01 μIU/mL, and the functional sensitivity was 0.011 μIU/mL. Intra-assay coefficient of variation (CV) was 5.64%. Subjects with values between 0.4 and 5.0 mIU/L were assumed to be euthyroid. TSH below 0.4 mIU/L was considered suppressed and TSH above 5.0 mIU/L was considered elevated. FT4 was measured using a two-step enzyme immunoassay (Access Free T4) with a reference range of 9.0–19.0 pmol/L. FT4 was measured with a presumptive view to identify the subjects with subclinical and overt thyroid dysfunction. The splitting up of the subjects in this way did not yield any useful information, and therefore, the data are not shown. Furthermore, the FT4 levels were used in the correlation analysis and as a factor in the regression analysis.

Serum total OPG was determined by a sandwich ELISA (BioVendor—Laboratorní medicína a.s.p, Czech Republic). OPG was expressed in pmol/L. The *analytical limit of detection* was 0.13 pmol/L. The *assay sensitivity* was 0.4 pmol/L. The intra-assay CV was 4.5%, the inter-assay CV was 5.5%, and the normal range (mean ± SEM) was 4.1 ± 2.3 pmol/L. OC was determined using a solid phase enzyme amplified sensitivity immunoassay (BioSource hOST-EASIA Kit, Biosource, Belgium). OC was expressed in ng/mL. The method measured intact OC only, and no discrimination of carboxylated and uncarboxylated OC was possible. The assay sensitivity was 0.4 ng/mL. The intra-assay CV was 1.0% and the inter-assay CV was 5.3%. The reference range was 5 to 25 ng/mL.

Statistical Analysis

The numerical data are presented as means and standard deviations or median and interquartile range. The categorical data are presented as proportions and 95% confidence interval. The normality of distribution was assessed by means of a Kolmogorov–Smirnov test. The Student *t*-test was applied for comparison of normally distributed data. The Mann–Whitney *U*-test and the Kruskal–Wallis test were used for comparison of non-normally distributed continuous variables and for groups with a low number of cases. Spearman's rho was used to test the correlations between numerical variables that did not follow the normal distribution. A multifactor regression analysis was also applied. Two-sided *P*-values below 0.05 were considered statistically significant. 95% confidence intervals were applied in all tests. The data processing was done using SPSS for Windows v. 13 (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the studied subjects stratified by their menstrual status are presented in Table 1. We found a significantly higher WC, BMI, and TSH in the postmenopausal group. The prevalence of abdominal obesity was significantly higher among the postmenopausal women than in the menstruating ones (Pearson Chi square $P < 0.001$). The characteristics of the subjects in the two thyroid function groups and the euthyroid control group are presented in Table 2.

OPG

The serum OPG levels were significantly higher in the postmenopausal women, and the difference was observed irrespective of the thyroid function (Table 2). The OPG levels were significantly higher in the subjects with suppressed TSH compared to the euthyroid controls, but only in the menstruating women. There was a trend toward a U-shaped curve also in

Table 2. Comparison between the characteristics of the menstruating and postmenopausal subjects, grouped by thyroid function. The Student *t*-test was applied for age, BMI, OPG, FT4, and OC, and Mann–Whitney *U*-test was used for all analyses in the low TSH group because of the small case number, as well as for WC and FT4. Kruskal–Wallis and median tests were applied to compare the OPG and OC levels among the thyroid function groups.

	AGE (YEARS)	BMI (kg/m^2)	WAIST (cm)	OPG (pmol/L)	OC (ng/dL)	TSH (mU/L)	FT4 (pmol/L)
Low TSH group							
Menstruating, n = 13	40.9 ± 6.1	23.7 ± 3.8	80.3 ± 12.9	4.26 ± 1.51‡	10.71 ± 4.42	0.11 ± 0.09	19.9 ± 13.6
PM, n=13	60.5 ± 6.1	27.3 ± 5.1	85.7 ± 11.8	5.76 ± 2.08	16.85 ± 7.29*	0.17 ± 0.11	18.0 ± 6.7
P	<0.001	0.048	0.243	0.034	0.044	0.153	0.756
Elevated TSH group							
Menstruating, n = 15	43.4 ± 14.2	26.3 ± 5.1	83.9 ± 13.3	4.11 ± 2.43	8.37 ± 6.42	10.44 ± 1.54	10.4 ± 1.8
PM, n = 34	61.6 ± 6.7	26.7 ± 4.9	88.7 ± 10.6	5.94 ± 0.94	10.76 ± 3.87*	14.48 ± 12.54	9.6 ± 3.0
P	<0.001	0.878	0.275	0.001	0.027	0.004	0.123
Euthyroid control group							
Menstruating, n = 36	37.7 ± 13.4	24.9 ± 4.7	79.8 ± 12.4	3.59 ± 1.94	8.38 ± 6.73	2.57 ± 0.11	12.0 ± 10.6
PM, n = 41	60.4 ± 8.3	28.4 ± 4.3	89.9 ± 13.2	5.77 ± 1.64	13.64 ± 3.23	2.44 ± 1.49	12.9 ± 1.8
P	<0.001	0.005	<0.001	<0.001	0.002	0.763	0.155

Notes: ‡$P = 0.048$; *$P = 0.024$.
Abbreviation: PM, postmenopausal.

Table 3. Correlations among the studied parameters.

MENSTRUAL STATUS			AGE (Y)	BMI (kg/m²)	WAIST (cm)	TSH (mU/L)	FT4 (pmol/L)
Menstruating N = 64	OPG (pmol/L)	*r*	0.31	0.19	0.16	0.08	−0.24
		P	0.015	0.142	0.220	0.529	0.117
	OC (ng/dL)	*r*	−0.02	−0.31	−0.15	−0.23	0.27
		P	0.876	0.019	0.269	0.091	0.083
PM N = 78	OPG (pmol/L)	*r*	0.57	−0.15	−0.02	0.09	0.15
		P	<0.001	0.195	0.848	0.421	0.283
	OC (ng/dL)	*r*	−0.02	−0.23	−0.42	−0.29	0.40
		P	0.898	0.032	<0.001	0.013	0.002

the menstruating group, with a lowest point in the euthyroid subgroup. In the postmenopausal women, OPG correlated strongly and positively with age, Spearman's rho = 0.57, $P < 0.001$ (Table 3). In the premenopausal women, the correlation with age was weaker (Spearman's rho = 0.31, $P = 0.015$). In neither of the two groups did OPG correlate with WC, BMI, FT4, or TSH, which was further confirmed by the multifactor regression analysis (Table 4). The only significant factor in the latter was the subjects' age.

OC

The OC levels were significantly higher in the postmenopausal group compared to the premenopausal one, irrespective of the thyroid function (Table 2). The OC levels were significantly lower in the postmenopausal women with elevated TSH than in those with suppressed TSH ($P = 0.047$). The levels in the euthyroid controls fell in between, but did not differ significantly from the two other groups. The differences within the menstruating group were not significant, either. OC correlated negatively with BMI and WC in the postmenopausal group (Spearman rho = −0.23, $P = 0.032$ and rho = −0.420, $P < 0.001$), but not with age (Table 3). In the premenopausal group, OC did not correlate with any of the studied parameters, except the BMI (Spearman's rho = 0.31, $P < 0.019$).

In the postmenopausal group, the OC levels correlated positively with FT4 (rho = 0.40, $P = 0.002$) and negatively with TSH (rho = −0.29, $P = 0.013$). The only significant factors for the OC levels in the multifactor regression analysis were the menopausal status and WC (Table 4).

Discussion

We observed higher OPG and OC levels in the postmenopausal women compared to those with preserved menstrual cycle. On the other hand, no parallel was found between OPG and OC with respect to their association with the other studied parameters in our cohort. The thyroid function was associated differently with the levels of the two proteins. OPG levels demonstrated a trend toward lower values in the euthyroid subjects compared to those with elevated or suppressed TSH. Our results are very close to the data reported by Botella-Carretero et al in a small group of thyroxin-treated thyroid cancer subjects.[18] It is noteworthy that the curves of both

Table 4. Results of the multiple regression analysis with osteocalcin and osteoprotegerin as independent variables.

	UNSTANDARDIZED COEFFICIENTS		STANDARDIZED COEFFICIENTS	t	SIG.
	B	STD. ERROR	BETA		
Osteocalcin					
(Constant)	15.985	3.455		4.626	<0.001
Age (years)	−0.031	0.053	−0.074	−0.583	0.56
TSH (mU/L)	−0.065	0.046	−0.117	−1.405	0.16
Menstrual status	5.384	1.490	0.463	3.614	<0.001
BMI (kg/m²)	−0.222	0.100	−0.188	−2.233	0.027
Osteoprotegerin					
(Constant)	−0.235	1.099		−0.214	0.83
Age (years)	0.112	0.017	0.691	6.560	<0.001
TSH (mU/L)	−0.009	0.016	−0.039	−0.559	0.58
Menstrual status	−0.348	0.489	−0.076	−0.712	0.48
BMI (kg/m²)	−0.012	0.033	−0.027	−0.376	0.71

OPG and OC in the two studies are quite similar despite the different settings: a longitudinal follow-up of a single group vs. a comparison of three subpopulations with a different thyroid function. Therefore, our work complements the already existent data. We observed, however, an opposite association with the subjects' menstrual status and age.

The mechanisms underlying the higher OPG and OC in the postmenopausal hyperthyroid subjects are still not very well understood. One possible explanation is the increase in bone turnover through the effect of TH and reduced TSH or by an altered cytokine production, as demonstrated in other studies.[17,20] The OPG increase in the hypothyroid state reported by other authors is even less clear. One possible explanation is the endothelial dysfunction observed in hypothyroid subjects, since bone remodeling is decreased in hypothyroidism and should not contribute to high OB-derived molecule level. A study by Martini et al demonstrated an increase in RANKL but not in OPG with the administration of recombinant TSH.[21] It is also suggested that TSH may exert a bone-sparing effect by the Wnt signaling pathway rather than by the OPG.[22] Moreover, it is generally unknown what proportion of the circulating OPG is derived from the bone or from other tissues, eg the vascular endothelium. OPG has been found to be elevated in subjects with atherosclerotic vascular disease, and to be linked to cardiovascular risk.[11] Some authors, however, question this relationship.[23] The increased bone turnover and the state of a mild chronic systemic inflammation with the menopause and aging, on the other hand, may explain the loss of the difference among the three thyroid function groups in the postmenopausal women. We performed a linear regression model in an attempt to assess the effect of multiple possibly interacting factors, especially as the postmenopausal subjects were significantly older than the menstruating ones. The only factor in the model that influenced significantly the OPG levels was age.

In our study, OC showed an almost linear positive relationship with the thyroid function. The increase in OC levels with increasing TH levels was expected, but we observed it only in the postmenopausal women, probably resulting from the more prominent effect of hyperthyroidism on bone remodeling after menopause.[24,25] The only significant factors associated with the OC levels in the regression analysis, however, were the menopausal status and WC. In the postmenopausal group, OC correlated negatively with WC and BMI. These findings can be interpreted to a certain extent in the light of previously published data.[26] WC is a measurement of abdominal obesity and is closely linked to insulin resistance.[27] Our results, therefore, support the hypothesis of an association between OC and insulin sensitivity. Lee et al found a negative association of OC with the markers of insulin resistance in an animal model, thus demonstrating a possible link that closes the loop between bone and energy metabolism.[28] Ferron et al[29] and Pittas et al[30] studied the markers of metabolic syndrome in human subjects and reached similar conclusions.

The markedly weaker negative correlation of OC with BMI may mean that OC is related to body composition through the insulin/tissue insulin sensitivity/visceral fat and not through the total fat mass, as suggested by Pepene et al,[31] who explored the potential role of OC in Polycystic ovary syndrome patients. A negative association of OC with the markers of insulin resistance in postmenopausal women has been reported also by Kanazawa et al.[32] The same authors observed a negative correlation of OC with intima-media thickness and adiposity in type 2 diabetic subjects. These findings, however, have been brought into question by the data published by Mori et al[33] and Schwartz et al,[34] which illustrate how complex and equivocal the subject is.

One possible explanation for the totally different associations of OC in the premenopausal and postmenopausal women observed by us is that the estrogens are a very strong determinant of body composition and bone turnover in women. After the decline of the ovarian estrogen production other mechanisms take over. García-Martín et al demonstrated a positive association of increased folicle stimulating hormone and luteinizing hormone with the markers of bone turnover in postmenopausal women.[35] The increased peri- and postmenopausal bone turnover is accompanied by a higher release of OC in the systemic circulation, which attenuates the insulin resistance and probably counteracts the trend toward hyperinsulinemia and increasing abdominal fat in aging females.[36,37] It can be speculated that in the setting of a profound estrogen deficiency, the relative homeostatic interactions between bone and energy metabolism gain more significance.

OPG is also affected by the menopausal transition. Unlike OC, its levels are influenced by age, and this was seen more prominently in the postmenopausal group. Whether this was merely the result of a nonlinearity of the association with an age threshold at which the slope becomes steeper is hard to conclude. Possibly this finding might be related to the increased cardiovascular morbidity in the postmenopausal women. It is still debatable whether estrogen deficiency is the sole cause of the steep increase in cardiovascular diseases in this group. Age might contribute independently by modulating the cellular effects of the sex steroids.[38] The higher prevalence of abdominal obesity, dyslipidemia, and arterial hypertension form a more prevalent "metabolic syndrome" phenotype in the postmenopausal women.

The current study has several limitations. The thyroid function subgroups are relatively small, especially the group with suppressed TSH. We did not separate the subjects with subclinical and overt thyroid dysfunction because the size of the groups would have dropped further and statistical processing would not have been applicable. The influence of thyroid function might be more demonstrable with larger groups. It would also be interesting to measure the fasting immunoreactive insulin levels and fasting blood glucose. Investigating OPG and OC would also be more informative if bone mineral

density and bone resorption markers are controlled for, which was not possible in the current study.

In conclusion, OPG and OC are affected by the interplay of multiple factors, with menopause in females being probably of primary significance. The thyroid function is associated with their levels differentially, but the effect is again modulated by the menopausal status. The observed inverse association of OC with abdominal obesity in the postmenopausal women and the increase of OPG with age in the same group might be linked to the cardiovascular risk in aging females. Therefore, further research is necessary to clarify such a relationship and its direction.

Acknowledgments

We would like to thank the following participants in the study: Kristina Pantcheva, Gergana Antalavitcheva, Tatiana Kornilova, Donka Bogilova, Elena Stavreva.

Author Contributions

Conceived and designed the experiments: AMB, AS, IA. Analyzed the data: AS. Wrote the first draft of the manuscript: AS. Contributed to the writing of the manuscript: AMB, RK. Agree with manuscript results and conclusions: RK, IA, JV, LD. Jointly developed the structure and arguments for the paper: AS, AMB. Made critical revisions and approved final version: AMB. All authors reviewed and approved of the final manuscript.

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